As researchers unravel the biochemical system by which cells register and respond to hypoxia, they're coming up with potential new strategies for treating cancer, heart attack, and stroke

How Cells Endure Low Oxygen

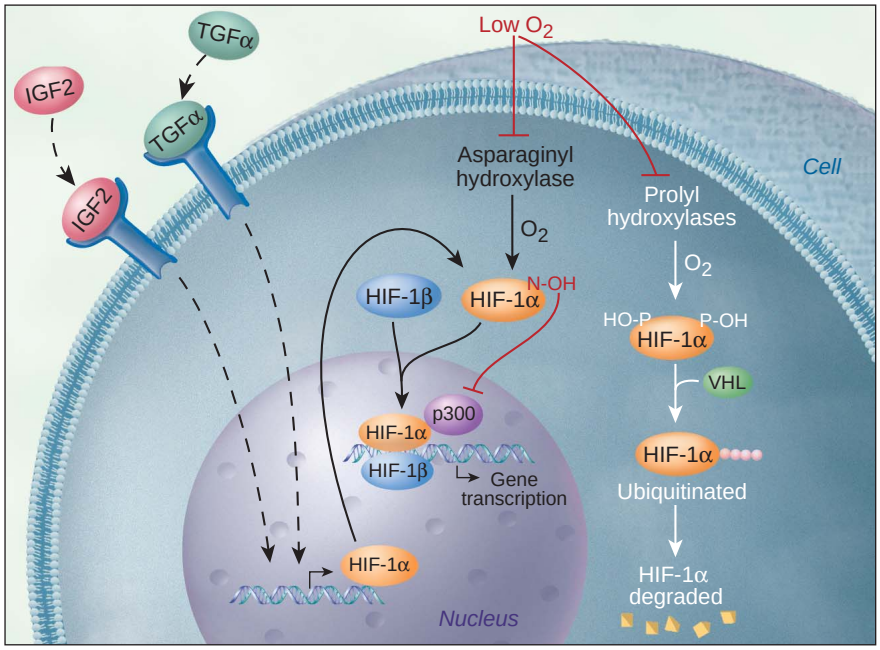
Cells can't exactly gasp for breath when they are deprived of oxygen. But they do have their own way of coping: a highly efficient system for turning on a host of genes that can counteract the effects of low oxygen levels, or hypoxia. Recently, researchers have learned a great deal about how this intricate system works, and the results have surprisingly broad implications: Drugs that target this system could potentially lead to better therapies for diseases from cancer to heart attacks to inflammation.

One key discovery revealed the heart of the system: a family of related proteins called hypoxia-inducible factors (HIFs). HIFs are transcription factors that turn up the activity of a variety of genes when oxygen becomes scarce, which can happen in a variety of both normal and pathological conditions. Some tissues, including the placenta and hard-working muscles, naturally have to deal with low oxygen concentrations, whereas in other cases—say, during a heart attack or stroke—HIFs can help mitigate the damage caused by a cutoff of blood flow to critical tissues.

The genes controlled by the HIFs include those coding for proteins that stimulate red blood cell production and angiogenesis, the formation of new blood vessels, as well as for glycolytic enzymes that can produce energy from glucose without the aid of oxygen. "HIF-1 is a sort of master switch that allows cells to respond to falling oxygen," says William Kaelin of Harvard's Dana-Farber Cancer Institute in Boston.

HIFs have also been linked to disease. Many kinds of cancers carry elevated levels of the proteins. This change may contribute to the cancers' ability to grow and spread, partly because it revs up angiogenesis, which in turn provides tumors with new blood vessels that fuel their growth. As a result, scientists in both industry and academia are looking for compounds to inhibit HIF activity. Conversely, drugs that increase HIF activity may be useful for treating victims of heart attacks and strokes.

Much more work will be needed, however, first to develop drugs that act on HIFs, and then to see if they are safe to give to patients. One indication of the enormous interest HIFs are generating: The first-ever conference on hypoxia will be held at the end of the month at a Keystone Symposium in Steamboat Springs, Colorado.



Dual paths. Cells control HIF-1 in two ways. When oxygen is plentiful, hydroxylase enzymes promote degradation of the HIF-1α subunit, which is aided by the VHL tumor suppressor protein, and also block HIF-1α's ability to bind p300 and other proteins needed for gene transcription. Low oxygen concentrations inhibit those activities of the hydroxylases, thus turning up HIF-1 activity. In addition, stimulation of growth control pathways by insulin growth factor 2 (IGF2) and transforming growth factor α (TGFα) lead to an increase in the activity of the *HIF-1α* gene, which can occur even at high oxygen concentrations.

ILLUSTRATION: CARIN CAIN/*SCIENCE*

A multitalented protein

Researchers first identified HIF-1 about 12 years ago, when they were trying to figure out how the body controls production of erythropoietin (EPO), a protein that stimulates production of red blood cells and is now widely used to prevent anemia in patients with chronic kidney failure. Oxygen deficiency stimulates EPO production, and Gregg Semenza's team at Johns Hopkins University School of Medicine in Baltimore, Maryland, had set out to discover how this happens. The researchers found that a particular sequence in the *EPO* gene plays a critical role, and when they then looked for proteins that bind to that sequence, they came up with HIF-1.

The protein's functions in the body have turned out to be surprisingly broad. Semenza estimates that perhaps 5% of the human genome comes under its control, although the exact suite of genes regulated varies depending on the cell type. In addition to genes involved in glycolysis and angiogenesis, HIF-1's targets include those involved in controlling cell growth, division, survival, and mobility. Whereas only a few cell types produce EPO, HIF-1 "worked in all cells, irrespective of whether they make EPO. That was extremely unexpected," says Peter Ratcliffe of the Henry Wellcome Building of Genomic Medicine at the University of Oxford, U.K.

Consistent with its broad impact on gene regulation, HIF-1 plays major roles both in embryonic development and in adults. The protein consists of two essential subunits,

called HIF-1α and HIF-1β. About 6 years ago, Semenza's team and also that of Randall Johnson at the University of California, San Diego, showed that knocking out the gene for the α subunit causes mouse embryos to die around the tenth day of gestation. The embryos' many abnormalities included defective blood vessel and heart development, presumably because normal angiogenesis doesn't occur without functional HIF-1.

Subsequent work by Celeste Simon's team at the University of Pennsylvania (Penn) School of Medicine in Philadelphia showed that HIF-1 is also needed for the placenta to establish normal blood connections in the uterine lining. "Virtually all aspects of the vascular system are fine-tuned by this [HIF] system," Simon says.

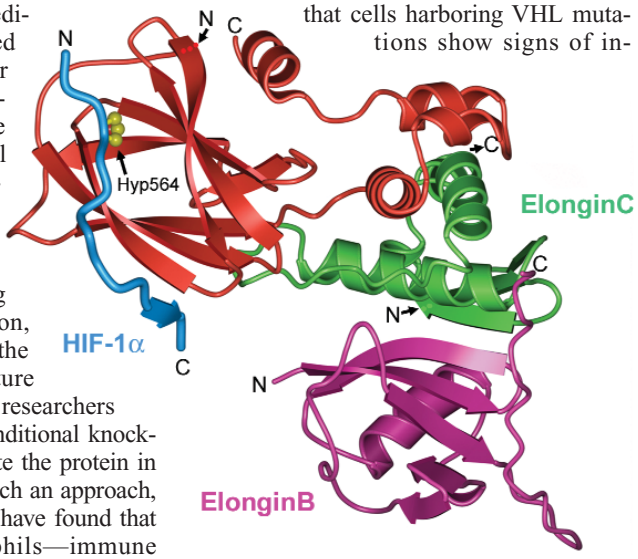
Because embryos lacking HIF-1 die early in gestation, they provide few clues to the protein's role in more mature tissues. Recently, however, researchers have produced so-called conditional knockouts, in which they inactivate the protein in specific cell types. Using such an approach, Johnson and his colleagues have found that macrophages and neutrophils—immune cells whose activity can lead to inflammation—depend on HIF-1, probably because they often operate in hypoxic conditions, such as in wounds and abscesses. "Knocking out HIF in macrophages and neutrophils basically blocks inflammation," Johnson says. One obvious implication: Drugs that inhibit HIF-1 activity might be useful for treating inflammation, he suggests.

Maintaining control

While researchers were making these discoveries, they were also finding that the body keeps HIF-1 activity under tight control. Within the past few years, they've started to figure out how that control is achieved, with a particular focus on HIF-1α as HIF's oxygen-sensitive component.

Early work showed that cells maintain relatively constant concentrations of the β subunit, but that HIF-1α concentrations vary, going up when oxygen levels are low and down when they are normal. These HIF-1α fluctuations are achieved partly by regulating destruction of the subunit. As shown by Jaime Caro of Jefferson Medical College in Philadelphia and others, when oxygen levels are normal, the protein is targeted for destruction by addition of a small protein called ubiquitin. But a fundamental question remained, says Kaelin: "How do cells know they are hypoxic and should leave HIF alone?"

A big clue came in 1999 when Ratcliffe's group showed that cells lacking the von Hippel-Lindau (VHL) tumor suppressor protein do not degrade HIF-1α properly. There was already good reason to suspect a link between HIF and VHL. Patients who have *VHL* gene mutations develop numerous cancers, particularly renal cell carcinomas—tumors that not only are copiously supplied with blood vessels but also have abnormally high levels of HIF-1. And Kaelin's group had previously found that cells harboring VHL mutations show signs of increased HIF activity. The reason for that became clear when Ratcliffe, Kaelin, and others showed that the VHL protein attaches ubiquitin to HIF-1α. The mutations prevent that addition, allowing HIF-1α to build up.



Targeted for destruction. The protein structure shows how a HIF-1α segment (blue), which contains a hydroxylated proline (Hyp564), binds to the pVHL protein (red). As a result, VHL and its partners, including ElonginB and ElonginC, add ubiquitin to HIF-1α, leading to its degradation.

The link to oxygen levels came in 2001, when three groups, one led by Ratcliffe and Christopher Schofield of the University of Oxford and the others by Kaelin and Frank Lee at the University of Pennsylvania School of Medicine, showed that HIF-1α must have hydroxyl groups tacked onto two specific residues of the amino acid proline before it can be ubiquitinated by VHL. Shortly thereafter, Ratcliffe's team and, independently, Steve McKnight and Richard Bruick of the University of Texas (UT) Southwestern Medical Center in Dallas identified the prolyl hydroxylase enzymes required for the hydroxylation reaction, which requires oxygen.

A second oxygen-dependent enzyme came into the picture the following year. Murray Whitelaw of the University of Adelaide in Australia, Bruick, and colleagues discovered that FIH-1, a naturally occurring HIF-1 inhibitor identified in Semenza's lab, uses oxygen to add hydroxyl groups to an asparagine residue near the carboxy end of HIF-1α. This prevents HIF-1α from interacting with other transcription factors to regulate gene activities.

Many, perhaps most, of the researchers working on HIFs now think that the prolyl and asparaginyl hydroxylases are themselves the "oxygen sensors" that keep HIF-1 turned off when cells have plenty of oxygen. "They provide a direct interface with molecular oxygen," Ratcliffe says.

But other researchers think the enzymes come into play later, in response to the actual sensor. This group includes Penn's Simon and Paul Schumacker of the University of Chicago, who have proposed that the sensor may instead be located in the mitochondria, the organelles that provide most of the cell's energy when oxygen is abundant.

They base this suggestion partly on their finding that cells lacking functional mitochondria don't respond to low oxygen levels by activating HIF. The missing ingredient in such cells may be the so-called reactive oxygen species (ROS) that mitochondria produce when oxygen concentrations are low. Schumacker's group has found that adding reactive oxygen in the form of hydrogen peroxide stabilizes HIF-1α. In contrast, overexpression of catalase, an enzyme that helps cells eliminate ROS, abolishes that response.

The issue of the oxygen sensor's identity is sure to be a hot topic at the upcoming Keystone Symposium. "The central question regarding HIF-1α is what the oxygen sensor is," Schumacker says.

The cancer connection

Oxygen-sparked degradation of HIF-1α isn't the only way cells regulate the protein's concentration, however. Work by Semenza, Amato Giaccia of Stanford University School of Medicine, and others has shown that activation of two of the major pathways that stimulate cell growth leads to increased production of the protein even when oxygen levels are normal. It's apparently a preemptive strategy that allows tissues—and tumors—to cope with decreased oxygen concentrations as they expand. This response can be buttressed, however, by inhibition of HIF-1α breakdown due to hypoxia. "The combination results in very high levels of [HIF-1α] expression," Semenza says.

Elevated HIF activity may contribute to the development of human cancers. HIF-1α has a relative, HIF-2α, which was discovered in the late 1990s by McKnight's team. Exactly what HIF-2α does is an outstanding question, although recent results from Joseph Garcia, also at UT Southwestern

CREDIT: J.-H. MIN *ET AL.*, *SCIENCE* **296**, 1886 (2002)

Medical Center, and his colleagues suggest that it may help protect cells against oxidative damage. Whatever it does, HIF-2α, like its cousin HIF-1α, has been linked to cancer.

Researchers have found that many human cancers, including common ones such as breast, colon, and lung cancers, have much higher levels of either HIF-1α or HIF-2α than do surrounding tissues. The changes in gene expression triggered by HIF-1 can promote cancer in a variety of ways. They foster the formation of new blood vessels, promote the spread of cancer cells to new sites by providing molecules needed for cell motility, and inhibit the programmed cell death that might otherwise eliminate abnormal cells.

Although HIF-2α's activities may be different, recent work by Kaelin's team shows that it is the chief culprit behind the growth of renal carcinoma cells in which the *VHL* gene is mutated. Normally, addition of the normal *VHL* gene to such cells inhibits their ability to form tumors when transplanted into mice—as would be expected for a tumor suppressor. But as the researchers reported in the December 2003 issue of the *Public Library of Science Biology*, this doesn't happen if renal carcinoma cells have an altered form of the *HIF-2α* gene that doesn't respond to *VHL*. Conversely, suppressing HIF-2α activity in the cells suppresses tumor growth.

Taken together, Kaelin says, the results show that suppression of HIF-2α is both necessary and sufficient to arrest the growth of these renal carcinomas. The work has caused a shift in his thinking. Previously, Kaelin says, "I thought that the tumor outgrowing its blood supply was causing up-regulation of *HIF*. Now I have to think it is the other way around"—that up-regulation of the *HIF* genes causes the tumors.

Less clear is whether HIFs alone can lead to the growth of cancers not linked to *VHL* mutations, although McKnight says they "probably have a very, very substantial role." Among other things, researchers including Semenza, Giaccia, and Adrian Harris of the Churchill Hospital in Oxford, U.K., have linked the elevated HIF levels seen in many cancers to a poor prognosis for patients. The finding is consistent with something oncologists have known for years: Hypoxia makes tumors resistant to both radiation and many chemotherapeutic drugs. "Hypoxic cells need three times as much radiation to kill as normally oxygenated cells," Giaccia says. What's more, he adds, "many chemotherapeutic agents target rapidly proliferating cells. Hypoxia inhibits proliferation."

The involvement of HIFs in cancer has touched off an intensive search for drugs that can prevent the proteins from performing their normal functions. The work is still in its early stages. So far, researchers have come up mainly with agents that work indirectly rather than by specifically inhibiting HIF. One such effort comes from Giovanni Melillo of the National Cancer Institute in Frederick, Maryland, and his colleagues. They screened roughly 2000 compounds for anti-HIF activity and identified four, including a known cancer drug, they reported in 2002 in *Cancer Research*. It's an inhibitor of the enzyme topoisomerase, which aids in the unwrapping of the DNA double helix during gene transcription. The drug may therefore work by blocking HIF's effects on gene activity.

Some other known anticancer drugs, including Herceptin and Gleevec, may work partly by inhibiting HIF action, but their effects are also likely to be indirect. For example, they may inhibit the growth pathways that stimulate HIF-1α synthesis.

Other researchers have come up with new drugs that target HIF. For example, Garth Powis of the Arizona Cancer Center in Tucson and his colleagues identified a compound called PX-478 that reduces HIF levels in cancer cells, although the mechanism is still unclear. The compound, he says, has "exceptional antitumor activity" in animal models. Powis, who started a company called Prolex to develop cancer drugs, hopes to move PX-478 into clinical trials soon.

Still, researchers prefer direct—and presumably more specific—HIF inhibitors that might have fewer side effects than drugs with broader actions. Those efforts are going slowly. "Targeting transcription factors [such as HIF] is attractive but very difficult," says Melillo. The problem is that it's harder to block protein-protein interactions than, say, the active site of an enzyme.

For that reason, it may be easier to develop drugs that bolster HIF action, which might be useful for treating conditions such as heart attack or stroke. Such drugs might be developed, for example, by finding inhibitors of the prolyl hydroxylases that mark HIF-1α for degradation.

Still, researchers note that many obstacles will have to be overcome before any drugs that target HIF find their way into the clinic. One recent note of caution comes from Johnson. Working with Gabriele Bergers's team at the University of California, San Francisco, he found that HIF-1α–deficient tumors that are derived from astrocytes, a type of neuronal support cell, behave differently depending on their location in the body. When transplanted under the skin of mice, an environment low in blood vessels, the tumors, as expected, grew poorly compared to those with active HIF-1. But when transplanted into the much more vessel-rich brain—where astrocytomas normally form—just the opposite occurred. The HIF-1α knockout cells grew even faster than those with the protein, and they spread much more extensively throughout the brain. The results suggest that clinicians will need to understand the precise biology of their patients' tumors before attempting anti-HIF therapy, although this is a problem for cancer therapies generally.

Even at best, however, blocking HIF activity may not be sufficient to wipe out tumors. Giaccia notes that in animal models anti-HIF therapy usually doesn't kill all the tumor cells, and the survivors can begin growing again. "Just inhibiting HIF may not be the panacea for cancer everyone thinks it will be," he says.

Finally, there are concerns about possible harmful side effects of drugs that target HIF, given the proteins' widespread effects in the body. Researchers hope that HIF inhibitors will be less toxic to normal cells, which usually have plenty of oxygen, than they are to tumor cells, which tend to be hypoxic, but that remains to be seen. Conversely, there are worries that giving HIF potentiators to stroke or heart attack victims might promote tumor growth.

Even with all the uncertainties about potential clinical applications, researchers are already pleased by what they've learned about how cells cope with hypoxia. "Almost everything about HIF makes sense," McKnight says. **–JEAN MARX**

Image not available for online use.

Inflammation promoter. Painting the skin of a normal mouse with an irritant causes inflammatory changes, including edema and immune cell infiltration in the skin (*left*). Inactivation of the *HIF-1α* gene (*right*) in macrophages and other immune cells prevents this response.

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